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PATENT

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FILING UNDER 37 C.F.R. 1.53(b)

Box Patent Application
Assistant Commissioner for Patents
Washington, DC 20231



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Sir:

This is a request for filing a **continuation** application under 37 C.F.R. 1.53(b)
of

Applicant(s): Thim et al.

Title: Human Spasmolytic Polypeptide in Glycosylated Form

39 pages of specification 9 sheets of drawings

3 sheets of Declaration and Power of Attorney

[x] The filing fee is calculated as follows:

Basic Fee:	\$690.00
Total Claims: $12 - 20 = 0 \times 18 =$	\$0
Independent Claims: $2 - 3 = 0 \times 78 =$	\$0
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Priority of Danish application no. 0068/93 filed on January 21, 1993 is claimed
under 35 U.S.C. 119.

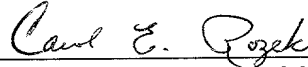
The benefit of application nos. 08/491,976 and 09/027,893 filed on August 2, 1995
and February 23, 1998 in the U.S. and PCT/DK94/00037 filed January 20, 1994 via the
PCT is claimed under 35 U.S.C. 120.

Address all future communications to Steve T. Zelson, Esq., Novo Nordisk of
North America, Inc., 405 Lexington Avenue, Suite 6400, New York, NY 10174-6401.

Please charge the required fee, estimated to be \$690, to Novo Nordisk of North America, Inc., Deposit Account No. 14-1447. A duplicate of this sheet is enclosed.

Respectfully submitted,

Date: March 20, 2000



Carol E. Rozek, Reg. No. 36,993
Novo Nordisk of North America, Inc.
405 Lexington Avenue, Suite 6400
New York, NY 10174-6401
(212) 867-0123

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

EXPRESS MAIL CERTIFICATE

Box Patent Application
Assistant Commissioner for Patents
Washington, DC 20231

Re: U.S. Patent Application for
Title: Human Spasmolytic Polypeptide in Glycosylated Form
Applicants: Thim et al.

Sir:

Express Mail Label No. EL473440171US

Date of Deposit : March 20, 2000

I hereby certify that the following attached paper(s) or fee

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Miriam Kelly
(Signature of person mailing paper(s) or fee)

Mailing Address:
Novo Nordisk of North America, Inc.
405 Lexington Avenue, Suite 6400
New York, NY 10017
(212) 867-0123

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Thim et al.

Serial No.: Not Yet Assigned

Group Art Unit: Not Yet Assigned

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Examiner: Not Yet Assigned

For: Human Spasmolytic Polypeptide in Glycosylated Form

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

Before the above-captioned application is taken up for examination, entry of the following amendment is respectfully requested:

IN THE SPECIFICATION:

At page 1, before the first line, insert

--CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a divisional of USSN 09/027,893 filed 23 February 1998, which is a continuation of USSN 08/491,976 filed August 2, 1995, now U.S. Patent No. 5,783,416, which is a continuation of PCT/DK94/00037 filed January 20, 1994, and claims priority under 35 U.S.C. 119 of Danish application serial no. 0068/93 filed January 21, 1993, which is incorporated herein by reference.--

IN THE CLAIMS:

Please cancel claims 1- 27 without prejudice or disclaimer. Please add new claims :

28. (New) Human spasmolytic polypeptide (hSP) characterized by being in glycosylated form.

29. (New) The hSP of claim 28 glycosylated is at Asn 15.

30. (New) The hSP of claim 28, wherein the glycosylated form comprises a glycosylated side chain comprising at least one hexose unit.

31. (New) The hSP of claim 30, wherein the glycosylated side chain comprises at least one mannose unit.

32. (New) The hSP of claim 31, wherein the glycosylated side chain comprises 13-17 mannose units.

33. (New) The hSP of claim 28, wherein the glycosylated form comprises at least one unit of N-acetyl glucosamine (GlcNAc).

34. (New) The hSP of claim 28, wherein the glycosylated form comprises (GlcNAc)₂(Man)₁₀₋₁₅.

35. (New) A method of preparing glycosylated human spasmolytic polypeptide (hSP), wherein said hSP has the amino acid sequence of SEQ ID NO:1 and is at least 60% glycosylated, comprising:

- (a) transforming a host cell with a DNA fragment encoding said hSP and capable of providing glycosylation of said hSP;
- (b) culturing said transformed cell under conditions permitting production of said hSP; and
- (c) recovering the resulting spasmolytic polypeptide from the culture.

36. (New) The method of claim 35, wherein the host cell is a yeast cell or a filamentous fungus cell.

37. (New) A pharmaceutical composition comprising the hSP of claim 28 together with a pharmaceutically acceptable carrier or excipient.

38. (New) A method for the treatment of gastrointestinal disorders, comprising administering the pharmaceutical composition of claim 37.


39. (New) The method of claim 36, wherein the host cell is a *Saccharomyces cerevisiae* or *Aspergillus oryzae*.

REMARKS

This amendment is submitted to cancel claims 1-27 and present new claims 28-39. No new matter added, and entry of the amendment is respectfully requested.

Respectfully submitted,

Date: 17 March 2000


Valeta A. Gregg, Reg. No. 35,127
Novo Nordisk of North America, Inc.
405 Lexington Avenue, Suite 6400
New York, NY 10174-6401
(212) 867-0123

HUMAN SPASMOLYTIC POLYPEPTIDE IN GLYCOSYLATED FORM

FIELD OF INVENTION

The present invention relates to human spasmolytic polypeptide in glycosylated form, variants of human and porcine spasmolytic polypeptides and a method of producing spasmolytic polypeptides in glycosylated form.

BACKGROUND OF THE INVENTION

Human spasmolytic polypeptide (HSP) belongs to a family of peptides containing one or more characteristic trefoil domains [1]. The trefoil domain is made up of a sequence of 38 or 39 amino acid residues in which 6 cystein residues are linked in the configuration 1-5, 2-4 and 3-6 thus forming a characteristic trefoil structure [1]. The trefoil family of peptides consists of rat intestinal trefoil factor, ITF [2], human breast cancer associated peptide, pS2 [3,4,5], porcine, human and murine spasmolytic polypeptide (PSP, HSP, MSP) [6,7,8] and frog spasmolysins (xP1, xP2 and xP4) [8,10,11] all containing 1, 2 or 4 trefoil domains (Fig. 1).

The physiological function of the trefoil peptides is poorly understood, and so far only PSP has been studied in any detail. In the porcine pancreas, PSP is found in the acinar cells and to be secreted in large amounts (50-100 mg/ml) into the pancreatic juice upon stimulation with pancreozymin or secretin [12,13,14]. PSP is resistant to digestion by intestinal proteases in the gastrointestinal tract [12], and specific binding of PSP to rat intestinal mucosa cells and membrane preparations from these cells has been demonstrated [15,16]. In the porcine gastrointestinal tract, specific receptor-like binding to Paneth cells in the duodenum has been found [17]. These results suggest a unique intraluminal function of the peptide. A pharmacological screening has indicated that PSP has spasmolytic and gastric acid secretion inhibitory effects [18],

and studies on mammalian cells have indicated a growth factor-like activity of PSP [19].

The DNA sequence and derived amino acid sequence of the human counterpart of porcine SP is shown in [8]. Unlike PSP, human SP 5 (Fig. 2), has been found to be expressed in the stomach, but not in the pancreas to any greater extent [8]. An increased expression of HSP and pS2 has been reported to be associated with peptic ulcers and mucosal injury in inflammatory bowel disease [20,21] indicating a possible healing function of these 10 peptides.

Only very limited amounts of HSP can be prepared by extraction of human tissue. An object of study resulting in the present invention was therefore to prepare recombinant HSP in sufficient amounts for physiological and biochemical studies of 15 the peptide.

SUMMARY OF THE INVENTION

It has surprisingly been found that when recombinant HSP is produced in certain host organisms, a proportion of it is produced in glycosylated form by posttranslational 20 modifications. The glycosylated form of HSP has not, to applicant's best knowledge, been described previously.

Accordingly, the present invention relates to human spasmolytic polypeptide (HSP) which has the amino acid sequence

Glu Lys Pro Ser Pro Cys Gln Cys Ser Arg Leu Ser Pro His Asn Arg
25 Thr Asn Cys Gly Phe Pro Gly Ile Thr Ser Asp Gln Cys Phe Asp Asn
Gly Cys Cys Phe Asp Ser Ser Val Thr Gly Val Pro Trp Cys Phe His
Pro Leu Pro Lys Gln Glu Ser Asp Gln Cys Val Met Glu Val Ser Asp
Arg Arg Asn Cys Gly Tyr Pro Gly Ile Ser Pro Glu Glu Cys Ala Ser
Arg Lys Cys Cys Phe Ser Asn Phe Ile Phe Glu Val Pro Trp Cys Phe
30 Phe Pro Asn Ser Val Glu Asp Cys His Tyr (SEQ ID NO:1)

or a functionally equivalent homologue thereof, characterized by being in glycosylated form.

In the present context, the term "functionally equivalent" is intended to indicate that the homologous polypeptide has a biological activity (e.g. spasmolytic effect) corresponding to that of native HSP. The term "homologue" is intended to indicate a polypeptide encoded by DNA which hybridizes to the same probe as the DNA coding for HSP under conditions of high or low stringency (e.g. as described in Sambrook et. al., 10 Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989). More specifically, the term is intended to refer to a DNA sequence which is at least 60% homologous to the sequence encoding HSP with the amino acid sequence shown above. The term is intended 15 to include modifications of the DNA sequence such as nucleotide substitutions which do not give rise to another amino acid sequence of the polypeptide, but which correspond to the codon usage of the host organism into which the DNA construct is introduced or nucleotide substitutions which do give rise to a 20 different amino acid sequence and therefore, possibly, a different protein structure which might give rise to a mutant polypeptide with different properties than the native enzyme. Other examples of possible modifications are insertion of one or more codons into the sequence, addition of one or more 25 codons at either end of the sequence, or deletion of one or more codons at either end or within the sequence. The term "glycosylated" is intended to indicate that a carbohydrate moiety is present at one or more sites of the protein molecule.

It is at present contemplated that glycosylation of HSP may 30 give rise to differences in the biological activity of the protein, for instance with respect to stability towards proteolytic enzymes in the gastrointestinal tract, solubility at gastric and/or intestinal pH compared to non-glycosylated HSP, antigenicity, half-life, tertiary structure, and targeting 35 to receptors on appropriate cells.

In another aspect, the present invention relates to a variant of a spasmolytic polypeptide (SP) which is a fragment of human spasmolytic polypeptide (HSP) or porcine spasmolytic polypeptide (PSP) comprising at least one trefoil domain.

5 The variant SP may be provided in both glycosylated and non-glycosylated form. It is at present contemplated that such a variant may be advantageous to use instead of full-length SP because of a higher specific biological activity, increased solubility and stability, longer half-life, easier way of
10 production, or the like.

It is assumed that other spasmolytic polypeptides than HSP will, if provided with a glycosylation site, also be expressed in predominantly glycosylated form. In a further aspect, the present invention therefore relates to a method of preparing a
15 spasmolytic polypeptide in at least 60% glycosylated form, wherein a host cell transformed with a DNA fragment encoding a spasmolytic polypeptide and capable of providing glycosylation of said spasmolytic polypeptide is cultured under conditions permitting production of said spasmolytic polypeptide and
20 recovering the resulting spasmolytic polypeptide from the culture.

DETAILED DESCRIPTION OF THE INVENTION

It has been found that, at least when recombinant HSP is produced in yeast, the proportion of it that is provided in
25 glycosylated form is in N-glycosylated form. It has further been found that glycosylation takes place at Asn15 of the sequence shown above. In preferred embodiments of glycosylated HSP, the glycosylated side chain contains at least one hexose unit. In particular, the glycosylated side chain may contain at
30 least one mannose unit, preferably at least five mannose units, most preferably at least ten mannose units. In one preferred embodiment of glycosylated HSP of the invention, the glycosylated side chain contains 13-17 mannose units. In other

preferred embodiments, the glycosylated HSP is in addition glycosylated with at least one unit of N-acetyl glucosamine (GlcNAc). In the currently preferred embodiment, the glycosylated HSP is glycosylated at Asn15 with (GlcNAc)₂(Man)₁₀.

5 15.

It is further contemplated to produce homologues of HSP which are provided with one or more additional glycosylation sites. Thus, the present invention also relates to HSP homologues, wherein Lys2 is replaced by Asn, Gln7 is replaced by Asn, Arg10 is replaced by Asn, Gly 20 is replaced by Thr or Ser, Gly23 is replaced by Asn, Ile 24 is replaced by Asn, Phe 36 is replaced by Asn, Asp 37 is replaced by Asn, Ser39 is replaced by Asn, Gln53 is replaced by Asn, Glu61 is replaced by Asn, Asp64 is replaced by Asn, Arg66 is replaced by Thr or Ser, Gly69 is replaced by Thr or Ser, Gly72 is replaced by Asn, Ile 89 is replaced by Thr or Ser, Pro98 is replaced by Asn or Val101 is replaced by Thr or Ser, or a combination of two or more of these substitutions. In a currently preferred embodiment of such an HSP homologue, Asp64 is replaced by Asn, and Arg66 is replaced by Thr or Ser.

It is of course understood that HSP homologues of the invention may be glycosylated in the same manner at one or more of these sites as described above for glycosylation at Asn15.

It is assumed that the trefoil structure common among spasmolytic polypeptides is important for the function of HSP and PSP. The variant human or porcine SP comprising a fragment of the full-length polypeptide should therefore include at least three disulfide bonds to provide this structure. Consequently, the variant may comprise at least a sequence of 30 amino acids from position 8 to 46 or from position 58 to 95, each of which sequences defines a trefoil domain of HSP and PSP.

As indicated above, the SP variant of the invention may be provided in non-glycosylated form. This may, for instance, be accomplished by substituting Asn15 by another amino acid, e.g. Asp or Glu, or by substituting Thr17 by another amino acid except Ser, e.g. Ala. It is more likely, however, that one or more additional glycosylation sites will be introduced into this domain, for instance by replacing Arg10 by Asn, Gly 20 by Thr or Ser, Gly23 by Asn, Ile 24 by Asn, Phe 36 by Asn, Asp 37 by Asn, or Ser39 by Asn, or a combination of two or more of these substitutions.

On the other hand, it may be desirable to provide the trefoil domain from position 58 to 95 with a glycosylation site lacking in this domain in native HSP and PSP. Thus, Glu61 may be replaced by Asn, Asp64 by Asn, Arg66 by Thr or Ser, Gly69 by Thr or Ser, or Gly72 is replaced by Asn, or a combination of two or more of these substitutions. In a currently preferred embodiment of the variant, Asp64 is replaced by Asn, and Arg66 is replaced by Thr or Ser.

It is of course understood that variants of the invention may be glycosylated in the same manner at one or more of these sites as described above for glycosylation at Asn15 in full-length HSP.

A DNA sequence encoding HSP may suitably be isolated from a human genomic DNA library by PCR (polymerase chain reaction) cloning using primers based on the published cDNA sequence [8]. Alternatively, the DNA sequence may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., EMBO Journal 3, 1984, pp. 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors. The cDNA sequence shown in [8] may be used as the basis of oligonucleotide synthesis.

Alternatively, it is possible to use cDNA coding for HSP obtained by screening a human cDNA library with oligonucleotide probes in accordance with well-known procedures.

Furthermore, the DNA sequence may be of mixed synthetic and 5 genomic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of genomic, synthetic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire DNA sequence, in accordance with standard techniques.

10 The SP variant of the invention may be encoded by a fragment of the full-length DNA sequence, prepared by one of the methods indicated above, or by suitably truncating the full-length sequence.

The DNA sequence encoding HSP or an SP variant of the invention 15 may then be inserted in a suitable expression vector. The recombinant expression vector may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an 20 autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and 25 replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding HSP or an SP variant of the invention should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which 30 shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA encoding

the inhibitor of the invention in mammalian cells are the SV 40 promoter (Subramani et al., Mol. Cell Biol. 1, 1981, pp. 854-864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science 222, 1983, pp. 809-814) or the adenovirus 2 major late promoter. Suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255, 1980, pp. 12073-12080; Alber and Kawasaki, J. Mol. Appl. Gen. 1, 1982, pp. 419-434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4, 599, 311) or ADH2-4c (Russell et al., Nature 304, 1983, pp. 652-654) promoters. Suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. 4, 1985, pp. 2093-2099) or the tpiA promoter.

The DNA sequence encoding HSP or an SP variant may also be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) or (for fungal hosts) the TPI1 (Alber and Kawasaki, op. cit.) or ADH3 (McKnight et al., op. cit.) promoters. The vector may further comprise elements such as polyadenylation signals (e.g. from SV 40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV 40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

The recombinant expression vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An examples of such a sequence (when the host cell is a mammalian cell) is the SV 40 origin of replication, or (when the host cell is a yeast cell) the yeast plasmid 2 μ replication genes REP 1-3 and origin of replication. The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or one which confers resistance to a drug, e.g. neomycin, hygromycin or

methotrexate, or the Schizosaccharomyces pombe TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130.

The procedures used to ligate the DNA sequences coding for HSP or the SP variant, the promoter and the terminator, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 1989).

- 10 The host cell into which the expression vector is introduced may be any cell which is capable of producing the inhibitor of the invention and is preferably a eukaryotic cell, such as a mammalian, yeast or fungal cell.

The yeast organism used as the host cell may be any yeast organism which, on cultivation, produces large quantities of the inhibitor of the invention. Examples of suitable yeast organisms are strains of the yeast species Saccharomyces cerevisiae, Saccharomyces kluyveri, Schizosaccharomyces pombe or Saccharomyces uvarum. The transformation of yeast cells may
20 for instance be effected by protoplast formation followed by transformation in a manner known per se.

Examples of suitable mammalian cell lines are the COS (ATCC CRL 1650), BHK (ATCC CRL 1632, ATCC CCL 10) or CHO (ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and
25 expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. 159, 1982, pp. 601-621; Southern and Berg, J. Mol. Appl. Genet. 1, 1982, pp. 327-341; Loyter et al., Proc. Natl. Acad. Sci. USA 79, 1982, pp. 422-426; Wigler et al., Cell 14, 1978, p. 725; Corsaro and
30 Pearson, Somatic Cell Genetics 7, 1981, p. 603, Graham and van der Eb, Virology 52, 1973, p. 456; and Neumann et al., EMBO J. 1, 1982, pp. 841-845.

Alternatively, fungal cells may be used as host cells. Examples of suitable fungal cells are cells of filamentous fungi, e.g. Aspergillus spp. or Neurospora spp., in particular strains of Aspergillus oryzae or Aspergillus niger. The use of Aspergillus spp. for the expression of proteins is described in, e.g., EP 238 023.

According to the present method, yeast cells are currently preferred for producing HSP and other SPs (such as those shown in Fig. 1), as they have surprisingly been found to produce SP 10 in a high yield and in at least 60% glycosylated form. For instance, about two thirds of the HSP produced by yeast may be recovered in glycosylated form.

The medium used to cultivate the cells may be any conventional medium suitable for growing mammalian cells or fungal 15 (including yeast) cells, depending on the choice of host cell. The spasmolytic polypeptide will be secreted by the host cells to the growth medium and may be recovered therefrom by conventional procedures including separating the cells from the medium by centrifugation or filtration, precipitating the 20 proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulfate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography or affinity chromatography, or the like.

The present invention also relates to a pharmaceutical 25 composition comprising HSP or a variant spasmolytic polypeptide of the invention together with a pharmaceutically acceptable carrier or excipient. In the composition of the invention, the variant may be formulated by any of the established methods of formulating pharmaceutical compositions, e.g. as described in 30 Remington's Pharmaceutical Sciences, 1985. The composition may typically be in a form suited for oral or rectal administration and may, as such, be formulated as tablets or suppositories.

HSP or an SP variant of the invention is contemplated to be useful for the prophylaxis or treatment of gastrointestinal disorders. More specifically, it is contemplated for the treatment of gastric or peptic ulcers, inflammatory bowel disease, Crohn's disease or injury to the intestinal tract caused by radiation therapy, bacterial or other infections, etc.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is further illustrated in the following example 10 with reference to the appended drawings in which

Fig. 1 shows the trefoil family of peptides. Intestinal trefoil factor (ITF) contains one trefoil domain [2], as does the breast cancer associated pS2 peptide [3,4]. The spasmolytic polypeptides from man, pig and mouse contain two trefoil domains [1,8]. Spasmolysins from Xenopus laevis contain one or four trefoil domains [10]. Recently, a member of the frog trefoil family containing two domains has been described [11].

Fig. 2 shows the proposed structure of human spasmolytic polypeptide, HSP. The primary amino acid sequence is taken from Tomasetto et al. [8], and the disulphide bonds are placed in homology to PSP [1].

Fig. 3 shows the nucleotide sequence (SEQ ID NO:2) and corresponding amino acid sequence (SEQ ID NO: 3) of the 563 bp EcoRI - XbaI fragment encoding the leader - HSP fusion protein. The Kex 2 processing site is indicated by a vertical arrow. The leader and the PCR cloned parts of the HSP gene are shown in capital letters, while the synthetic parts are shown in small letters. The underlined sequences correspond to the PCR primers with horizontal arrows indicating the direction. Restriction sites relevant for the construction are shown.

Fig. 4 shows the S. cerevisiae plasmid for the expression and secretion of HSP. TPI-prom. and TPI-term. are S. cerevisiae triosephosphate isomerase transcription promoter and terminator sequences, respectively. POT is a selective marker, the 5 Schizosaccharomyces pombe triosephosphate isomerase gene. Only restriction sites relevant for the construction of the plasmid have been indicated.

Fig. 5 shows reversed-phase HPLC on a Vydac 214TP54 column of yeast fermentation broth. The two peaks corresponding to r-HSP 10 and glycosylated r-HSP are indicated. The dashed line shows the concentration of acetonitrile in the eluting solvent.

Fig. 6 shows ion exchange chromatography on a Fast Flow S column of concentrated yeast supernatant. The amount of r-HSP and glycosylated r-HSP were determined by the use of the HPLC 15 system shown in Fig. 5. The bars indicate the fractions pooled for further purification of r-HSP and glycosylated r-HSP. The dashed line shows the concentration of NaCl in the eluting solvent. For details, see Material and Methods.

Fig. 7 shows the final purification of r-HSP (A) and 20 glycosylated r-HSP (B) on a preparative reversed-phase HPLC Vydac 214TP1022 column. The bars indicate the fractions pooled for lyophilization. The dashed lines show the concentration of acetonitrile in the eluting solvent. For details, see Material and Methods.

25 Fig. 8 shows reversed-phase HPLC on a Vydac 214TP54 column of purified, glycosylated r-HSP (A) and r-HSP (B). The dashed lines show the concentration of acetonitrile in the eluting solvent.

Fig. 9 shows mass spectra of purified r-HSP (A and B) and 30 glycosylated r-HSP (C and D). Fig. A and Fig. C show the original mass spectrum of r-HSP and glycosylated r-HSP, respectively. Fig. B and Fig. D show the reconstructed mass

spectrum for r-HSP and glycosylated r-HSP on the basis of Fig. A and Fig. C.

EXAMPLE

MATERIAL AND METHODS

5 General methods

Standard DNA techniques were used as previously described [29]. Synthetic oligonucleotides were prepared on an automatic DNA synthesizer (380B, Applied Biosystems) using commercially available reagents. DNA sequence determinations were performed
10 by the dideoxy chain-termination technique [30]. Polymerase chain reactions (PCR) were performed on a DNA Thermal Cycler (Perkin Elmer Cetus) using a commercial kit (GeneAmp, Perkin Elmer Cetus).

PCR cloning of HSP

15 The first trefoil domain of HSP was isolated by a PCR reaction in which 1 μ g human genomic DNA (Clontech, Palo Alto, CA, USA) was used as a template. The reaction mixture contained 100 pmole each of the forward primer 1 (GGCTGAGCCCCCATAACAG) (SEQ ID NO:4) and reverse primer 2 (TGGAACACCAGGGGAC) (SEQ ID NO:5)
20 (Fig. 3) and was carried out in a 100 μ l volume. The cycle was : 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. After 30 cycles a final cycle was performed in which the 72°C step was maintained for 10 min. The PCR product, a 115 bp fragment, was isolated by electrophoresis on a 2% agarose gel.

25 The 115 bp PCR fragment was digested with DdeI and then ligated to a 31 bp duplex formed from the oligonucleotides (GAGAAACCCTCCCCCTGCCAGTGCTCCAGGC) (SEQ ID NO:6) and (TCAGCCTGGAGCACTGGCAGGGGGAGGGTTTCTC). The ligation product was amplified by PCR using forward primer 3
30 (GCTGAGAGATTGGAGAAGAGAGAGAAACCCTCCCCCT) (SEQ ID NO:7) and reverse primer 2. The 3' part of primer 3 is identical to the

N-terminal encoding part of the HSP gene and the 5' part of primer 3 is identical to the C-terminal encoding part of the hybrid leader gene (Fig. 3). In-frame fusion of the hybrid leader gene and the first trefoil domain from HSP was obtained 5 by overlay extension PCR [31]. The product was digested with EcoRI and AvaII and isolated as a 360 bp DNA fragment.

The second trefoil domain of HSP was PCR-cloned from human genomic DNA as described for the first domain by replacing primers 1 and 2 with forward primer 4 (TGCGTCATGGAGGTCTC) (SEQ ID NO:8) and reverse primer 5 (AGCACCATGGCACTTCAAAG) (SEQ ID NO:9) (Fig. 3). Reverse primer 5 introduces a NcoI site as a silent mutation. The PCR product, a 115 bp fragment, was isolated and digested with DdeI and NcoI resulting in a 91 bp fragment. To this fragment were ligated two synthetic duplexes. 15 The first, encoding the amino acid sequence between the two trefoil domains, consisted of the oligonucleotides (GTCCCCTGGTGTTTCCACCCCCTCCCAAAGCAAGAGTCGGATCAGTGCATGGAGGTC) (SEQ ID NO:10) and (TGAGACCTCCATGACGCACTGATCCGACTCTTGCT-TTGGGAGGGGGTGGAAACACCAGGG) (SEQ ID NO:11). The second, a 46 bp 20 NcoI - XbaI fragment encoding the C-terminal part of HSP, consisted of the oligonucleotides (CATGGTGCTTCTTCCCGAACTCTGT-GGAAGACTGCCATTACTAAGT) (SEQ ID NO:12) and (CTAGACTTAGTAATGGCAGTCTTCCACAGAGTTCGGGAAGAAGCAC) (SEQ ID NO:13). After AvaII digestion a 195 bp AvaII - XbaI fragment 25 was isolated.

A DNA construct encoding the hybrid leader fused in-frame to the entire HSP gene was obtained by ligation of the 360 bp EcoRI - AvaII fragment and the 195 bp AvaII - XbaI fragment described above to the 2.7 kb EcoRI - XbaI fragment from vector 30 pTZ19R [32]. This construct was then transformed into *E. coli* strain MT-172 (r^- , m^+) by selection for resistance to ampicillin. DNA sequencing of the resulting plasmid, KFN-1843, showed that the correct construction had been obtained.

Construction of the HSP secreting yeast strain

Plasmid KFN-1843 described above was digested with EcoRI and XbaI. The resulting 558 bp fragment was isolated and ligated to the 9.3 kb NcoI - XbaI fragment and the 1.6 kb NcoI - EcoRI 5 fragment both from the yeast expression vector pMT-636. Plasmid pMT-636 is derived from the S. cerevisiae - E. coli shuttle vector CPOT [25,33] by deletion of the 0.4 kb HpaI - NruI fragment from the Leu-2 gene. The ligation mixture was transformed into E. coli strain MT-172, and the HSP expression 10 plasmid, KFN-1847, was isolated (Fig. 4). Plasmid pKFN-1847 was transformed into S. cerevisiae strain MT-663 by selection for growth on glucose as the sole carbon source. One transformant, KFN-1852, was selected for fermentation.

Fermentation

15 The transformant described above was cultivated at 30°C for 3 days in yeast peptone dextrose (YPD) medium [40] supplied with additional yeast extract (60 g/l). An OD 650 nm value of 52 was reached at the end of the fermentation.

Purification of r-HSP

20 The concentration of r-HSP in the yeast fermentation broth and fractions obtained during the purification was measured by analytical HPLC. Aliquots (usually 50-200 µl) were injected onto a Vydac 214TP54 reverse-phase C4 HPLC column (0.46 x 25 cm) equilibrated at 30°C at a flow rate of 1.5 ml/min with 0.1% 25 (v/v) TFA in 5% (v/v) acetonitrile. The concentration of acetonitrile in the eluting solvent was raised to 65% (v/v) over 30 min. Absorbance was measured at 280 nm. The peaks eluting at 15.6 min. and 16.1 min. (Fig. 5) was found by mass spectrometry analysis to represent glycosylated r-HSP and 30 unglycosylated r-HSP, respectively. The peptides were quantified using a calibrated PSP sample as standard as both

peptides contain two Trp and two Tyr out of 106 amino acid residues.

From a 10 litre fermentor, 8 litres of fermentation broth was isolated by centrifugation at 3,000 rpm for 10 min. The 5 supernatant was concentrated to 0.9 litre using an Amicon ultrafiltration unit (RA 2000) equipped with an Amicon spiral ultrafiltration cartridge type S1Y3, MW cutoff 3,000 (Product No. 540620). The pH was adjusted to 1.7 and the conductivity in the resulting concentrated sample was measured to 4.7 mS.

- 10 The sample was pumped onto a Fast Flow S-Sepharose (Pharmacia) column (5 x 11 cm) with a flow rate of 40 ml/h. Previous to the application, the column was equilibrated in 50 mM formic acid buffer, pH = 3.7. After application of the sample, the column was washed with 500 ml of 50 mM formic acid buffer, pH = 3.7.
- 15 The peptides were eluted from the column by a linear gradient between 1.5 litres of 50 mM formic acid buffer, pH = 3.7 and 1.5 litres of 50 mM formic acid buffer, pH = 3.7 containing 0.6 M NaCl. Fractions of 10 ml was collected at a flow rate of 40 ml/h and the absorbance was measured at 280 nm. Fractions were
- 20 assayed for the content of r-HSP and glycosylated r-HSP in the HPLC-system previously described. The elution profile is shown in Fig. 6. Fractions corresponding to r-HSP (fract. Nos. 107-128) and glycosylated r-HSP (fract. Nos. 78-95), respectively, were pooled.
- 25 Glycosylated r-HSP and r-HSP were further purified by preparative HPLC chromatography. Pooled fractions (approx. 200 ml) were pumped onto a Vydac 214TP1022 C4 column (2.2 x 25 cm) equilibrated in 0.1% (v/v) TFA. The column was washed with 100 ml of 0.1% (v/v) TFA in 10% (v/v) MeCN. The peptides were
- 30 eluted at 25°C and at a flow rate of 5 ml/min with a linear gradient (650 ml) formed from MeCN/H₂O/TFA (10.0:89.9:0.1 v/v/v) and MeCN/H₂O/TFA (60.0:39.9:0.1 v/v/v). UV-absorption was monitored at 280 nm, and fractions corresponding to 10 ml were collected and analysed for the content of r-HSP or glycosylated

r-HSP. Fig. 7 shows the preparative HPLC purification of r-HSP (Fig. 7A) and glycosylated r-HSP (Fig. 7B). Fractions corresponding to the bars were pooled, and the volume reduced to 30% by vacuum centrifugation. From the two resulting pools, r-5 HSP and glycosylated r-HSP were isolated by lyophilization.

Characterization of r-HSP and glycosylated r-HSP

Amino acid composition analysis were carried out by hydrolysis of 50 µg peptide with 6M HCl for 24 h at 110°C as previously described [6]; no correction for loss during hydrolysis was carried out. Amino acid sequence analysis was determined by automated Edman degradation using an Applied Biosystems Model 470A gas-phase sequencer [22]. Carbohydrate composition analysis was carried out by hydrolysis of 50 µg peptide with 2M HCl for 1h, 2h and 4h at 100°C and monosaccharides were separated on a CarboPac PAI (Dionex, Sunnyvale, CA) column (4 x 250 mm) eluted with 14 mM NaOH. The monosaccharides were detected by pulsed amperometric detection (Dionex PAD-detector). The amount of monosaccharides was corrected to zero time of hydrolysis and calculated as nmol of monosaccharide per 20 nmol of peptide.

Mass spectrometry analysis was performed using an API III LC/MS/MS system (Sciex, Thornhill, Ontario, Canada). The triple quadrupole instrument has a mass-to-charge (m/z) range of 2400 and is fitted with a pneumatically assisted electrospray (also referred to as ion-spray) interface [23,24]. Sample introduction was done by a syringe infusion pump (Sage Instruments, Cambridge, MA) through a fused capillary (75 µm i.d.) with a liquid flow-rate set at 0.5-1 µl/min. The instrument m/z scale was calibrated with the singly-charged ammonium adduct ions of poly(propylene glycols) (PPG's) under unit resolution.

The accuracy of mass measurements was generally better than 0.02%.

RESULTS

Expression and purification

DNA fragments encoding the two trefoil domains of HSP were isolated by PCR from human genomic DNA using primers based on the published cDNA sequence [8]. The full length HSP gene was obtained from the PCR cloned fragments by addition of synthetic DNA fragments. The HSP gene was fused in-frame to a hybrid yeast leader sequence by overlay extension PCR [31] (Fig. 3). The hybrid leader is based on the mouse salivary amylase signal peptide [34] and the S. kluyveri α mating factor leader sequence [35] and is further modified near the Kex 2 cleavage site for efficient processing [36, 41].

The yeast expression plasmid pKFN-1847 contains the leader-HSP gene inserted between the S. cerevisiae triose phosphate isomerase promoter and terminator [37]. The expression vector (Fig. 4) also contains the Schizosaccharomyces pombe TPI gene (POT) [38].

The plasmid was transformed into the yeast strain MT-663, carrying a deletion in the TPI gene, by selecting for growth on glucose.

The expression level of r-HSP in the present yeast system is approx. 120 mg/l. As can be seen from Fig. 5, the yeast supernatant contains two forms of r-HSP; one eluting at $R_t = 15.6$ min. and one eluting at $R_t = 16.1$ min. These two forms were purified separately, and by using the analytical HPLC-system (Fig. 5), these two forms can be quantified individually during the different steps of the purification.

After the initial concentration of the yeast supernatant by ultrafiltration, the first purification step was cationic exchange chromatography on a Fast Flow S column. Fig. 6 shows the elution profile from the column including the amount of r-

HSP and glycosylated r-HSP determined in the fractions. A complete separation of the two forms of r-HSP was obtained in this step.

The fractions from the Fast Flows S column were pooled as indicated in Fig. 6, and the two peptides were further purified by preparative HPLC (Fig. 7). The r-HSP and glycosylated r-HSP were recovered from the fractions indicated in Fig. 7A and Fig. 7B by vacuum centrifugation and lyophilization. The purification is summarized in Table 1. The overall yield of r-HSP and glycosylated r-HSP from 8 litres of fermentation broth was 160 mg and 219 mg corresponding to 50% and 34%, respectively.

Characterization of r-HSP and glycosylated r-HSP

Fig. 8 shows the purity of r-HSP and glycosylated r-HSP as analysed by analytical HPLC. From these results none of the peptides looks completely pure. However, upon rechromatography of material eluting in the minor as well as the major peak, similar chromatograms were obtained for both peptides (results not shown). This seems to indicate that the double peak observed for both r-HSP and glycosylated r-HSP reflects an atypical behaviour of these peptides on reverse phase columns rather than impurities in the preparations.

Table 2 shows the amino acid sequencing results obtained on r-HSP and glycosylated r-HSP. The average repetitive yield was 94.4% (r-HSP) and 94.6% (glycosylated r-HSP), respectively. In both cases the first 40 residues of the two peptides were confirmed by the sequence analysis. In the glycosylated HSP, no PTH-a.a. was found in Edman degradation cycle No. 15. The HSP sequence from residue 15-17 (Asn-Arg-Thr) corresponds to a consensus sequence for N-glycosylation of Asn-15.

The carbohydrate composition analysis of glycosylated r-HSP showed the presence of 12.8 nmol mannose (Man) and 1.6 nmol of

N-acetyl glucosamine (GlcNAc) per nmol of r-HSP. By peptide mapping of r-HSP and glycosylated r-HSP in combination with mass spectrometry and sequencing analysis (results not shown), no other residue besides Asn-15 of the glycosylated r-HSP was found to be modified, i.e. no O-glycosylation was found.

In Fig. 9, the electro-spray mass spectrometry (ESMS) analysis is shown for r-HSP and glycosylated r-HSP. Fig. 9A and 9C are original mass spectra displaying characteristic series of multiply charged protonated ions always observed in ESMS spectra of proteins. Fig. 9B and 9D are the corresponding computer reconstructed mass spectra from which the molecular weight of individual components may be read directly. As can be seen from Fig. 9B, the MW found for r-HSP is 11961.5 ± 2 which is in very good agreement with a calculated mass of 11961.3. Fig. 9D shows the reconstructed ion spray mass spectrum of the glycosylated r-HSP. From the sequence analysis and the carbohydrate composition analysis, it is known that only Asn-15 is glycosylated and that only two monosaccharide residues, mannose and N-acetyl glucosamine, occur in the glycosylated form of r-HSP. From these results in combination with the mass spectrometry data, it is possible to deduce the different glycosylated forms of r-HSP (Table 3).

Molecular weights corresponding to two series of carbohydrate side chains can be deduced from the combination of carbohydrate composition data and ISMS-data, namely $(\text{GlcNAc})_2(\text{Hex})_{10-15}$ and $(\text{Hex})_{13-17}$ (Table 3). As mannose is the only hexose in the glycosylated r-HSP, and as Asn-15 is the only glycosylated residue, it seems reasonable to conclude that the structure of the glycosylation site is $\text{Asn}-(\text{GlcNAc})_2-(\text{Man})_{10-15}$. The observed $(\text{Hex})_{13-17}$ forms are thus most likely to arise from fragmentation in the mass spectrometer, by which the two GlcNAc residues lose an acetyl group and are converted into two hexoses.

The structure of Asn-(GlcNAc)₂-(Man)₁₀₋₁₅ has previously been reported as high mannose type of N-glycosylation for other peptides and proteins expressed in yeast [26].

Table 1

5 Purification of r-hSP and glycosylated r-hSP from yeast supernatant

STEP	Volume [ml]	Amount [mg]		Yield [%]	
		r-hSP	glycosylated r-hSP	r-hSP	glycosylated r-hSP
Yeast supernatant	8000	320	640	100	100
Ultrafiltration	900	207	405	65	63
Ion exchange chromatography	Pool 1	160	275		43
	Pool 2	220	182	57	
Prep HPLC	Pool 1	54	219		34
	Pool 2	80	160	50	

Table 2

Amino acid sequence analysis of r-hSP and glycosylated r-hSP

Cycle No.	PTH-a.a.	Yield (pmol)	
		r-hSP	glycosylated r-hSP
1	Glu	4304	8853
2	Lys	6925	8292
3	Pro	6027	12837
4	Ser	2890	5602
5	Pro	4336	8802
6	(Cys)	ND	ND
7	Gln	3388	5689
8	(Cys)	ND	ND
9	Ser	1279	2417
10	Arg	1876	2523
11	Leu	2277	4290
12	Ser	877	1790
13	Pro	1545	2963
14	His	517	574
15	Asn	1202	0*
16	Arg	959	1471
17	Thr	978	2172
18	Asn	1066	1509
19	(Cys)	ND	ND
20	Gly	836	1857
21	Phe	993	1958
22	Pro	843	1839
23	Gly	785	2049
24	Ile	640	1400
25	Thr	589	1454
26	Ser	274	621
27	Asp	581	1391
28	Gln	445	952
29	(Cys)	ND	ND
30	Phe	623	1562
31	Asp	483	1210
32	Asn	369	823
33	Gly	359	885
34	(Cys)	ND	ND
35	(Cys)	ND	ND
36	Phe	422	1094
37	Asp	268	783
38	Ser	127	324
39	Ser	145	394
40	Val	298	827

ND: Not determined

*: No trace of PTH-Asn or PTH-Asp was seen in cycle No. 15 of glycosylated r-hSP.

Table 3

Mass analysis of glycosylated r-hSP

Structure	Calculated MW	MW found by ESMS (Fig.9D)
hSP + 2 GlcNAc + 10 Man	13989.1	13989.5
hSP + 2 GlcNAc + 11 Man	14151.2	14151.0
hSP + 2 GlcNAc + 12 Man	14313.4	14313.5
hSP + 2 GlcNAc + 13 Man	14475.5	14475.0
hSP + 2 GlcNAc + 14 Man	14639.7	14639.5
hSP + 2 GlcNAc + 15 Man	14799.8	14801.5
hSP + 13 Man	14069.1	14072.0
hSP + 14 Man	14231.3	14232.5
hSP + 15 Man	14393.4	14393.0
hSP + 16 Man	14555.5	14557.5
hSP + 17 Man	14717.7	14720.0

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15
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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Novo Nordisk A/S
- (B) STREET: Novo Alle
- (C) CITY: Bagsvaerd
- (E) COUNTRY: Denmark
- (F) POSTAL CODE (ZIP): 2880
- (G) TELEPHONE: +45 4444 8888
- (H) TELEFAX: +45 4449 3256
- (I) TELEX: 37304

(ii) TITLE OF INVENTION: Human Spasmolytic Polypeptide in Glycosylated Form

(iii) NUMBER OF SEQUENCES: 14

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 106 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Glu Lys Pro Ser Pro Cys Gln Cys Ser Arg Leu Ser Pro His Asn Arg
1 5 10 15

Thr Asn Cys Gly Phe Pro Gly Ile Thr Ser Asp Gln Cys Phe Asp Asn
20 25 30

29

Gly	Cys	Cys	Phe	Asp	Ser	Ser	Val	Thr	Gly	Val	Pro	Trp	Cys	Phe	His
	35						40					45			
Pro	Leu	Pro	Lys	Gln	Glu	Ser	Asp	Gln	Cys	Val	Met	Glu	Val	Ser	Asp
	50					55					60				
Arg	Arg	Asn	Cys	Gly	Tyr	Pro	Gly	Ile	Ser	Pro	Glu	Glu	Cys	Ala	Ser
65					70				75						80
Arg	Lys	Cys	Cys	Phe	Ser	Asn	Phe	Ile	Phe	Glu	Val	Pro	Trp	Cys	Phe
				85					90					95	
Phe	Pro	Asn	Ser	Val	Glu	Asp	Cys	His	Tyr						
		100						105							

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 563 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 77..553

(ix) FEATURE:

- (A) NAME/KEY: sig_peptide
- (B) LOCATION: 77..235

(ix) FEATURE:

- (A) NAME/KEY: mat_peptide
- (B) LOCATION: 236..553

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile	
-53 -50 -45	
GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG	157
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu	
-40 -35 -30	

30

ATT COG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn -25 -20 -15	205
GTC GCC ATG GCT GAG AGA TTG GAG AAG AGA GAG AAA CCC TCC CCC TGC Val Ala Met Ala Glu Arg Leu Glu Lys Arg Glu Lys Pro Ser Pro Cys -10 -5 1 5	253
CAG TGC TCC AGG CTG AGC CCC CAT AAC AGG ACG AAC TGC GGC TTC OCT Gln Cys Ser Arg Leu Ser Pro His Asn Arg Thr Asn Cys Gly Phe Pro 10 15 20	301
GGA ATC ACC AGT GAC CAG TGT TTT GAC AAT GGA TGC TGT TTC GAC TCC Gly Ile Thr Ser Asp Gln Cys Phe Asp Asn Gly Cys Cys Phe Asp Ser 25 30 35	349
AGT GTC ACT GGG GTC CCC TGG TGT TTC CAC CCC CTC CCA AAG CAA GAG Ser Val Thr Gly Val Pro Trp Cys Phe His Pro Leu Pro Lys Gln Glu 40 45 50	397
TOG GAT CAG TGC GTC ATG GAG GTC TCA GAC AGA AGA AAC TGT GGC TAC Ser Asp Gln Cys Val Met Glu Val Ser Asp Arg Arg Asn Cys Gly Tyr 55 60 65 70	445
COG GGC ATC AGC CCC GAG GAA TGC GCC TCT CGG AAG TGC TGC TTC TCC Pro Gly Ile Ser Pro Glu Glu Cys Ala Ser Arg Lys Cys Cys Phe Ser 75 80 85	493
AAC TTC ATC TTT GAA GTG CCA TGG TGC TTC TTC COG AAC TCT GTG GAA Asn Phe Ile Phe Glu Val Pro Trp Cys Phe Phe Pro Asn Ser Val Glu 90 95 100	541
GAC TGC CAT TAC TAAGTCTAGA Asp Cys His Tyr 105	563

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 159 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser -35 -30 -25
Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Glu -20 -15 -10

31

Arg Leu Glu Lys Arg Glu Lys Pro Ser Pro Cys Gln Cys Ser Arg Leu
 -5 1 5 10
 Ser Pro His Asn Arg Thr Asn Cys Gly Phe Pro Gly Ile Thr Ser Asp
 15 20 25
 Gln Cys Phe Asp Asn Gly Cys Cys Phe Asp Ser Ser Val Thr Gly Val
 30 35 40
 Pro Trp Cys Phe His Pro Leu Pro Lys Gln Glu Ser Asp Gln Cys Val
 45 50 55
 Met Glu Val Ser Asp Arg Arg Asn Cys Gly Tyr Pro Gly Ile Ser Pro
 60 65 70 75
 Glu Glu Cys Ala Ser Arg Lys Cys Cys Phe Ser Asn Phe Ile Phe Glu
 80 85 90
 Val Pro Trp Cys Phe Phe Pro Asn Ser Val Glu Asp Cys His Tyr
 95 100 105

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

- (vi) ORIGINAL SOURCE:
- (A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GGCTGAGCCC CCATAACAG

19

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

- (vi) ORIGINAL SOURCE:
- (A) ORGANISM: synthetic

32

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TGGAAACACC AGGGGAC

17

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GAGAAACCCCT CCCCCGCCA GTGCTCCAGG C

31

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TCAGCCTGGA GCACTGGCAG GGGGAGGGTT TCTC

34

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

33

- (vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GCTGAGAGAT TGGAGAAGAG AGAGAAACCC TCCCCCT

37

- (2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TGOGTCATGG AGGTCTC

17

- (2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AGCACCATGG CACTTCAAAG

20

- (2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

34

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GTCCCCCTGGT GTTTCACCCC CCTCCCAAAG CAAGAGTCGG ATCAGTGGGT CATGGAGGTC

60

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 60 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

TGAGACCTCC ATGAOCCACT GATCOGACTC TTGCTTTGGG AGGGGGTGGG AACACCAGGG

60

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 46 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CATGGTGCTT CTTCCCGAAC TCTGTGGAAG ACTGCCATTG CTAAGT

46

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 46 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CTAGACTTAG TAATGGCAGT CTTCCACAGA GTTCGGGAAG AAGCAC

10771 10772 10773 10774 10775 10776 10777 10778 10779 10780 10781 10782 10783 10784 10785 10786 10787 10788 10789 10790 10791 10792 10793 10794 10795 10796 10797 10798 10799 10800 10801 10802 10803 10804 10805 10806 10807 10808 10809 10810 10811 10812 10813 10814 10815 10816 10817 10818 10819 10820 10821 10822 10823 10824 10825 10826 10827 10828 10829 10830 10831 10832 10833 10834 10835 10836 10837 10838 10839 10840 10841 10842 10843 10844 10845 10846 10847 10848 10849 10850 10851 10852 10853 10854 10855 10856 10857 10858 10859 10860 10861 10862 10863 10864 10865 10866 10867 10868 10869 10870 10871 10872 10873 10874 10875 10876 10877 10878 10879 10880 10881 10882 10883 10884 10885 10886 10887 10888 10889 10890 10891 10892 10893 10894 10895 10896 10897 10898 10899 10900 10901 10902 10903 10904 10905 10906 10907 10908 10909 10910 10911 10912 10913 10914 10915 10916 10917 10918 10919 10920 10921 10922 10923 10924 10925 10926 10927 10928 10929 10930 10931 10932 10933 10934 10935 10936 10937 10938 10939 10940 10941 10942 10943 10944 10945 10946 10947 10948 10949 10950 10951 10952 10953 10954 10955 10956 10957 10958 10959 10960 10961 10962 10963 10964 10965 10966 10967 10968 10969 10970 10971 10972 10973 10974 10975 10976 10977 10978 10979 10980 10981 10982 10983 10984 10985 10986 10987 10988 10989 10990 10991 10992 10993 10994 10995 10996 10997 10998 10999 11000 11001 11002 11003 11004 11005 11006 11007 11008 11009 11010 11011 11012 11013 11014 11015 11016 11017 11018 11019 11020 11021 11022 11023 11024 11025 11026 11027 11028 11029 11030 11031 11032 11033 11034 11035 11036 11037 11038 11039 11040 11041 11042 11043 11044 11045 11046 11047 11048 11049 11050 11051 11052 11053 11054 11055 11056 11057 11058 11059 11060 11061 11062 11063 11064 11065 11066 11067 11068 11069 11070 11071 11072 11073 11074 11075 11076 11077 11078 11079 11080 11081 11082 11083 11084 11085 11086 11087 11088 11089 11090 11091 11092 11093 11094 11095 11096 11097 11098 11099 11100 11101 11102 11103 11104 11105 11106 11107 11108 11109 11110 11111 11112 11113 11114 11115 11116 11117 11118 11119 11120 11121 11122 11123 11124 11125 11126 11127 11128 11129 11130 11131 11132 11133 11134 11135 11136 11137 11138 11139 11140 11141 11142 11143 11144 11145 11146 11147 11148 11149 11150 11151 11152 11153 11154 11155 11156 11157 11158 11159 11160 11161 11162 11163 11164 11165 11166 11167 11168 11169 11170 11171 11172 11173 11174 11175 11176 11177 11178 11179 11180 11181 11182 11183 11184 11185 11186 11187 11188 11189 11190 11191 11192 11193 11194 11195 11196 11197 11198 11199 11200 11201 11202 11203 11204 11205 11206 11207 11208 11209 11210 11211 11212 11213 11214 11215 11216 11217 11218 11219 11220 11221 11222 11223 11224 11225 11226 11227 11228 11229 11230 11231 11232 11233 11234 11235 11236 11237 11238 11239 11240 11241 11242 11243 11244 11245 11246 11247 11248 11249 11250 11251 11252 11253 11254 11255 11256 11257 11258 11259 11260 11261 11262 11263 11264 11265 11266 11267 11268 11269 11270 11271 11272 11273 11274 11275 11276 11277 11278 11279 11280 11281 11282 11283 11284 11285 11286 11287 11288 11289 11290 11291 11292 11293 11294 11295 11296 11297 11298 11299 11300 11301 11302 11303 11304 11305 11306 11307 11308 11309 11310 11311 11312 11313 11314 11315 11316 11317 11318 11319 11320 11321 11322 11323 11324 11325 11326 11327 11328 11329 11330 11331 11332 11333 11334 11335 11336 11337 11338 11339 11340 11341 11342 11343 11344 11345 11346 11347 11348 11349 11350 11351 11352 11353 11354 11355 11356 11357 11358 11359 11360 11361 11362 11363 11364 11365 11366 11367 11368 11369 11370 11371 11372 11373 11374 11375 11376 11377 11378 11379 11380 11381 11382 11383 11384 11385 11386 11387 11388 11389 11390 11391 11392 11393 11394 11395 11396 11397 11398 11399 11400 11401 11402 11403 11404 11405 11406 11407 11408 11409 11410 11411 11412 11413 11414 11415 11416 11417 11418 11419 11420 11421 11422 11423 11424 11425 11426 11427 11428 11429 11430 11431 11432 11433 11434 11435 11436 11437 11438 11439 11440 11441 11442 11443 11444 11445 11446 11447 11448 11449 11450 11451 11452

CLAIMS

1. Human spasmolytic polypeptide (HSP) which has the amino acid sequence

Glu Lys Pro Ser Pro Cys Gln Cys Ser Arg Leu Ser Pro His Asn Arg
5 Thr Asn Cys Gly Phe Pro Gly Ile Thr Ser Asp Gln Cys Phe Asp Asn
Gly Cys Cys Phe Asp Ser Ser Val Thr Gly Val Pro Trp Cys Phe His
Pro Leu Pro Lys Gln Glu Ser Asp Gln Cys Val Met Glu Val Ser Asp
Arg Arg Asn Cys Gly Tyr Pro Gly Ile Ser Pro Glu Glu Cys Ala Ser
Arg Lys Cys Cys Phe Ser Asn Phe Ile Phe Glu Val Pro Trp Cys Phe
10 Phe Pro Asn Ser Val Glu Asp Cys His Tyr (SEQ ID NO:1)

or a functionally equivalent homologue thereof, characterized by being in glycosylated form.

2. HSP according to claim 1, characterized by being in N-glycosylated form.

15 3. HSP according to claim 1 or 2, which is glycosylated at Asn15.

4. HSP according to claim 3, in which the glycosylated side chain contains at least one hexose unit.

5. HSP according to claim 4, in which the glycosylated side
20 chain contains at least one mannose unit, preferably at least five mannose units, most preferably at least ten mannose units.

6. HSP according to claim 5, in which the glycosylated side chain contains 13-17 mannose units.

7. HSP according to claim 4, which in addition is glycosylated
25 with at least one unit of N-acetyl glucosamine (GlcNAc).

8. HSP according to claim 7, which is glycosylated with (GlcNAC)₂(Man)₁₀₋₁₅.

9. HSP according to claim 1, wherein Lys2 is replaced by Asn, Gln7 is replaced by Asn, Arg10 is replaced by Asn, Gly 20 is replaced by Thr or Ser, Gly23 is replaced by Asn, Ile 24 is replaced by Asn, Phe 36 is replaced by Asn, Asp 37 is replaced by Asn, Ser39 is replaced by Asn, Gln53 is replaced by Asn, Glu61 is replaced by Asn, Asp64 is replaced by Asn, Arg66 is replaced by Thr or Ser, Gly69 is replaced by Thr or Ser, Gly72 is replaced by Asn, Ile 89 is replaced by Thr or Ser, Pro98 is replaced by Asn or Val101 is replaced by Thr or Ser, or a combination of two or more of these substitutions.

10. HSP according to claim 9, wherein Asp64 is replaced by Asn, and wherein Arg66 is replaced by Thr or Ser.

11. HSP according to claim 9, in which the glycosylated Asn contains at least one hexose unit.

12. HSP according to claim 11, which is glycosylated with at least one mannose unit, preferably at least five mannose units, most preferably at least ten mannose units.

13. HSP according to claim 12, which is glycosylated with 13-17 mannose units.

14. HSP according to claim 11, which in addition is glycosylated with at least one unit of N-acetyl glucosamine (GlcNAc).

15. HSP according to claim 14, which is glycosylated with (GlcNAc)₂(Man)₁₀₋₁₅.

16. A variant of a spasmolytic polypeptide which is a fragment of human spasmolytic polypeptide (HSP) or porcine spasmolytic polypeptide (PSP) comprising at least one trefoil domain.






17. A variant according to claim 16, which comprises at least a sequence of amino acids from position 8 to 46 or from position 58 to 95.
18. A variant according to claim 16 or 17, wherein Asn15 is substituted by another amino acid, e.g. Asp or Glu.
19. A variant according to claim 16 or 17, wherein Thr17 is substituted by another amino acid except Ser, e.g. Ala.
20. A variant according to claim 16 or 17, wherein Arg10 is replaced by Asn, Gly 20 is replaced by Thr or Ser, Gly23 is replaced by Asn, Ile 24 is replaced by Asn, Phe 36 is replaced by Asn, Asp 37 is replaced by Asn, Ser39 is replaced by Asn, Glu61 is replaced by Asn, Asp64 is replaced by Asn, Arg66 is replaced by Thr or Ser, Gly69 is replaced by Thr or Ser, or Gly72 is replaced by Asn, or a combination of two or more of these substitutions.
21. A method of preparing a spasmolytic polypeptide in at least 60% glycosylated form, wherein a host cell transformed with a DNA fragment encoding a spasmolytic polypeptide and capable of providing glycosylation of said spasmolytic polypeptide is cultured under conditions permitting production of said spasmolytic polypeptide and recovering the resulting spasmolytic polypeptide from the culture.
22. A method according to claim 21, wherein the host cell is a fungal cell such as a yeast cell, e.g. a strain of Saccharomyces cerevisiae, or a filamentous fungus cell, e.g. a strain of Aspergillus, such as Aspergillus oryzae.
23. A pharmaceutical composition comprising HSP according to any of claims 1-15 together with a pharmaceutically acceptable carrier or excipient.

24. A pharmaceutical composition comprising a variant spasmolytic polypeptide according to any of claims 16-20 together with a pharmaceutically acceptable carrier or excipient.

5 25. Use of HSP according to any of claims 1-15 for the manufacture of a medicament for the prophylaxis or treatment of gastrointestinal disorders.

26. Use of a variant spasmolytic polypeptide according to any of claims 16-20 for the manufacture of a medicament for the
10 prophylaxis or treatment of gastrointestinal disorders.

Trefoil family of peptides

Peptide	Number of trefoil domains	Species
Intestinal Trefoil Factor (ITF)		Rat Human
Breast cancer associated peptide (pS2)		Human
Spasmolytic polypeptide (hSP) (PSP) (mSP)		Human Porcine Mouse
Spasmolysins (xP1)		Frog
(xP4)		Frog

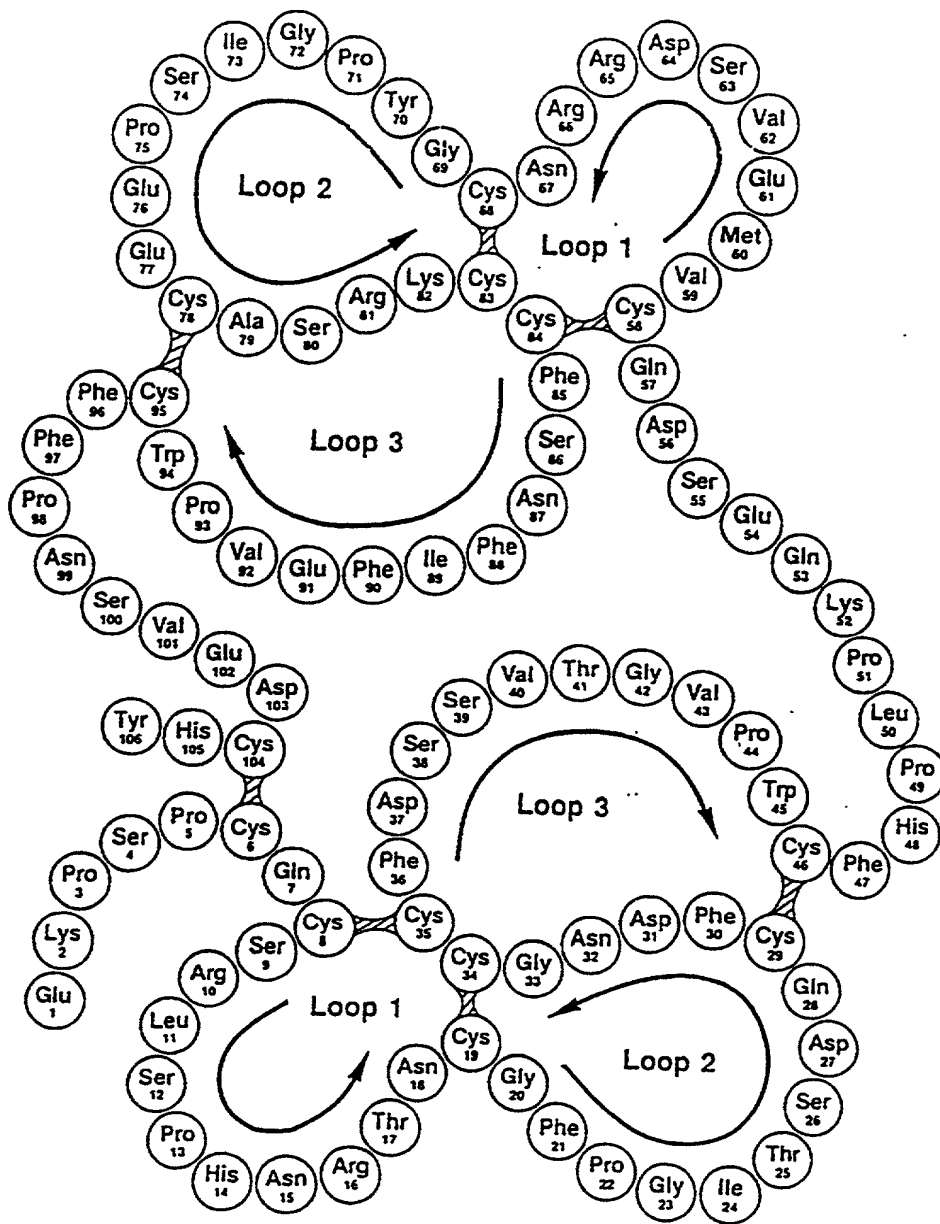


Fig. 2

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EcoRI

GAATTCCATTCAAGAATAGTTCAAACAAGAAGATTACAAACTATCAATTTCATACACAAT

ATAAACGACCAAAAGAATGAAGGCTGTTTTCTTGGTTTTGTCCTTGATCGGATTCTGCTG

M K A V F L V L S L I G F C W

GGCCCAACCAGTCACTGGCGATGAATCATCTGTTGAGATTCCGGAAGAGTCTCTGATCAT

A Q P V T G D E S S V E I P E E S L I I

CGCTGAAAACACCACTTTGGCTAACGTCGCCATGGCTGAGAGATTGGAGAAGAGAgagaa

A E N T T L A N V A M A E R L E K R E K

Dde I1→

accctccccctgcccagtgctccagggcTGAGCCCCCATAACAGGACGAAC TGCGGCTTCCC

P S P C Q C S R L S P H N R T N C G F P

TGGAATCACCAGTGACCAGTGTTTTGACAATGGATGCTGTTTCGACTCCAGTGTCAGTGG

G I T S D Q C F D N G C C F D S S V T G

AvaII← 24→

Ggtccccctggtggtttccacccccctcccaaagcaagagtcggatcagtgcggtcatggaggt

V P W C F H P L P K Q E S D Q C V M E V

Dde I

cTCAGACAGAAGAAACTGTGGCTACCCGGGCATCAGCCCCGAGGAATGCGCCTCTCGGAA

S D R R N C G Y P G I S P E E C A S R K

← 5Nco I

GTGCTGCTTCTCCAACCTTCATCTTTGAAGTGCCatggtgcttcttcccgaactctgtgga

C C F S N F I F E V P W C F F P N S V E

Xba I

agactgccattactaagtCTAGA

D C H Y

Fig. 3

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hSP expression vector

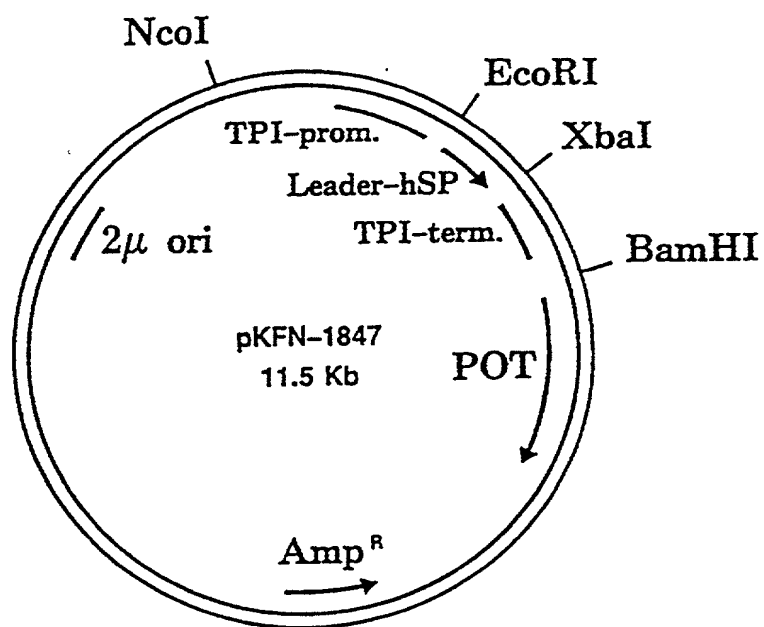


Fig. 4

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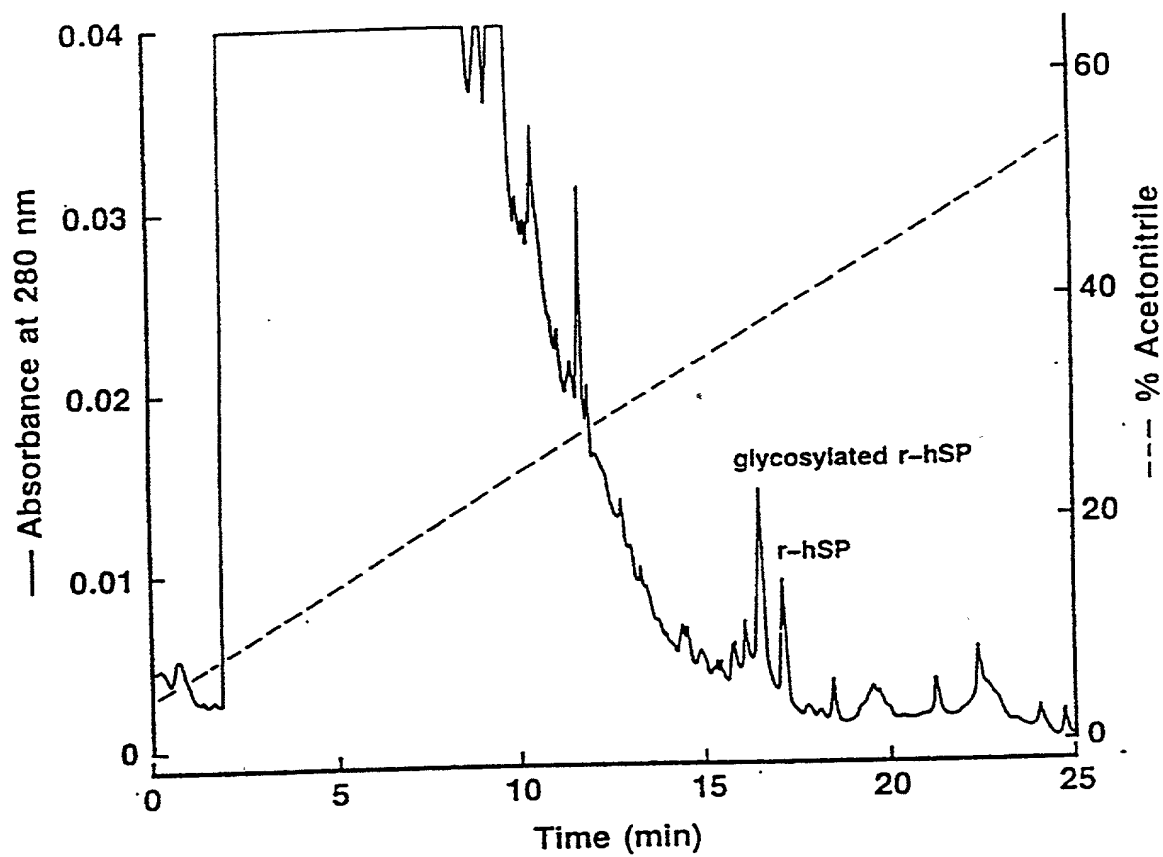


Fig. 5

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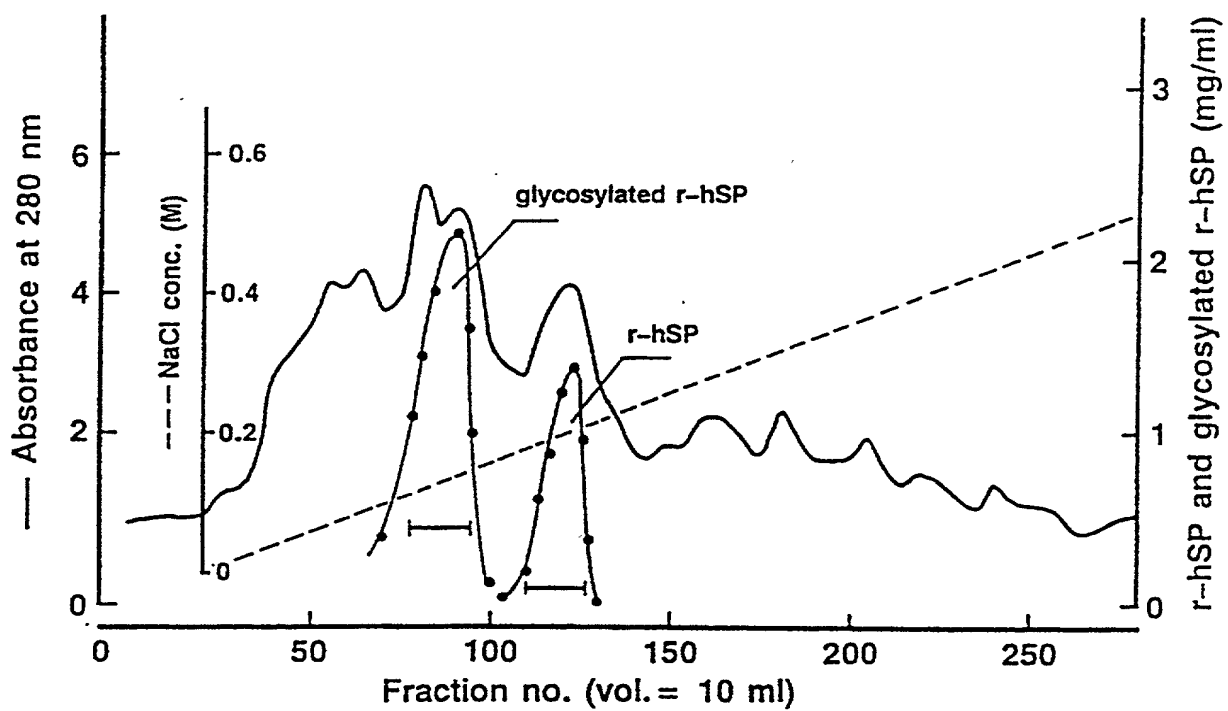


Fig. 6

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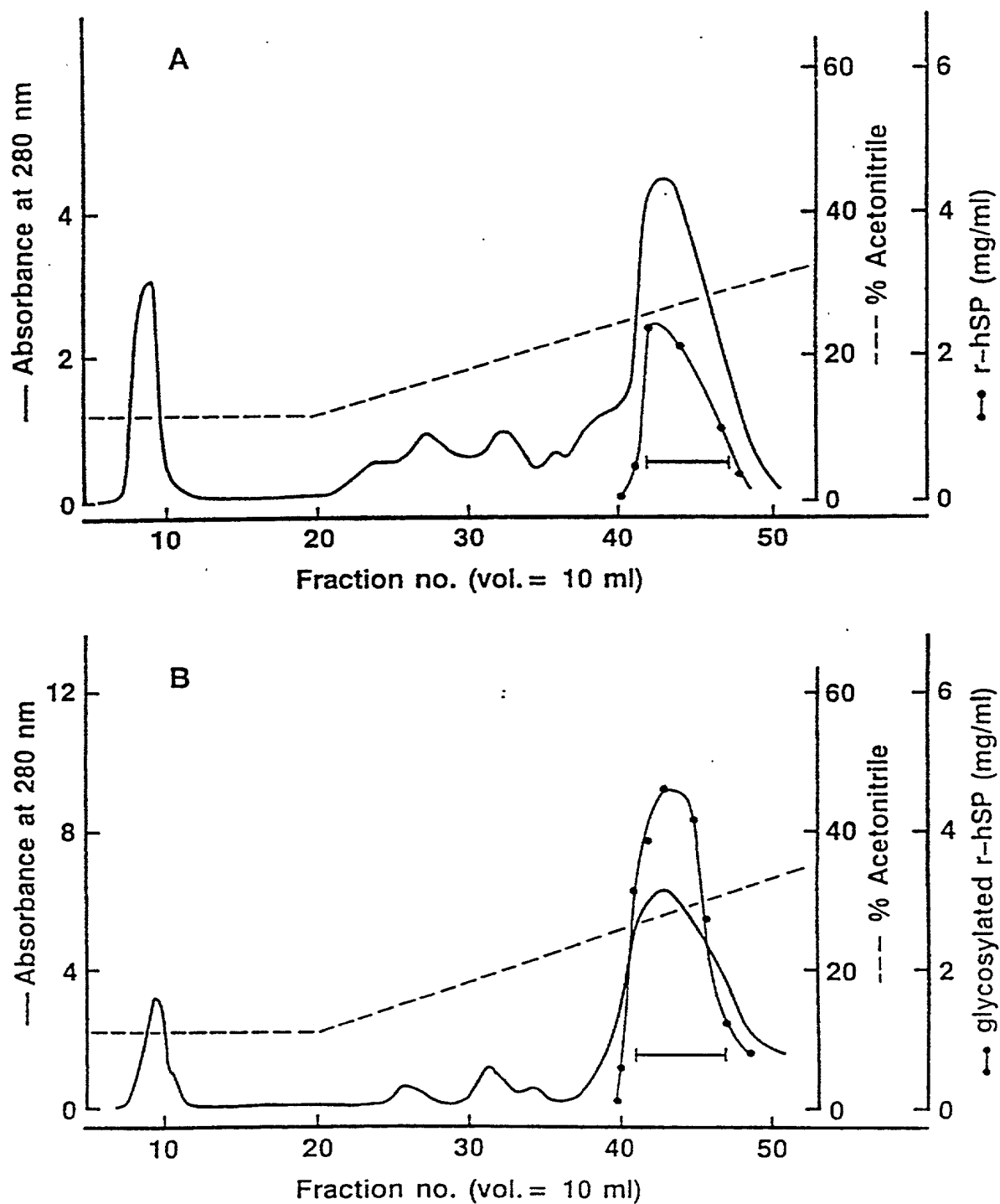


Fig. 7

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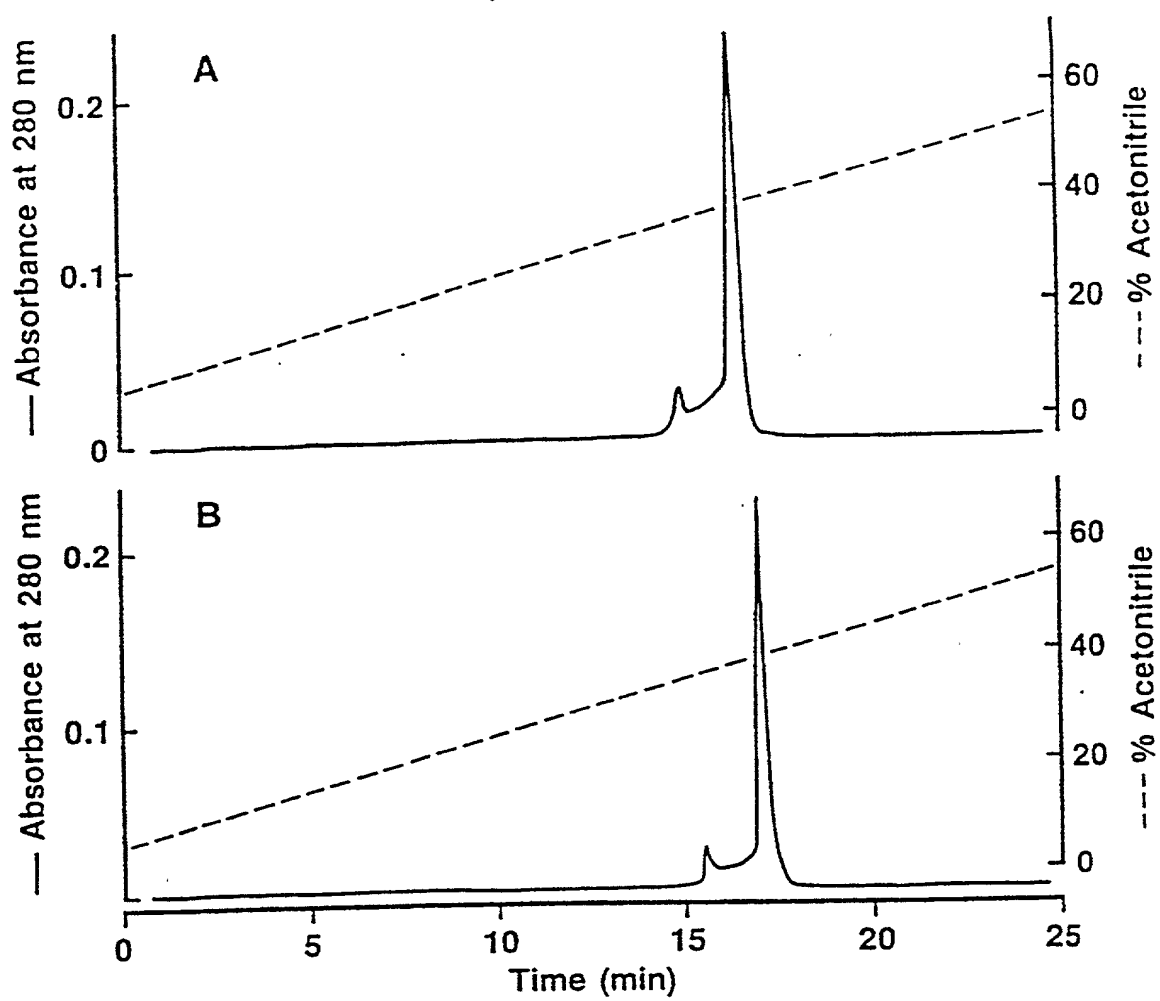
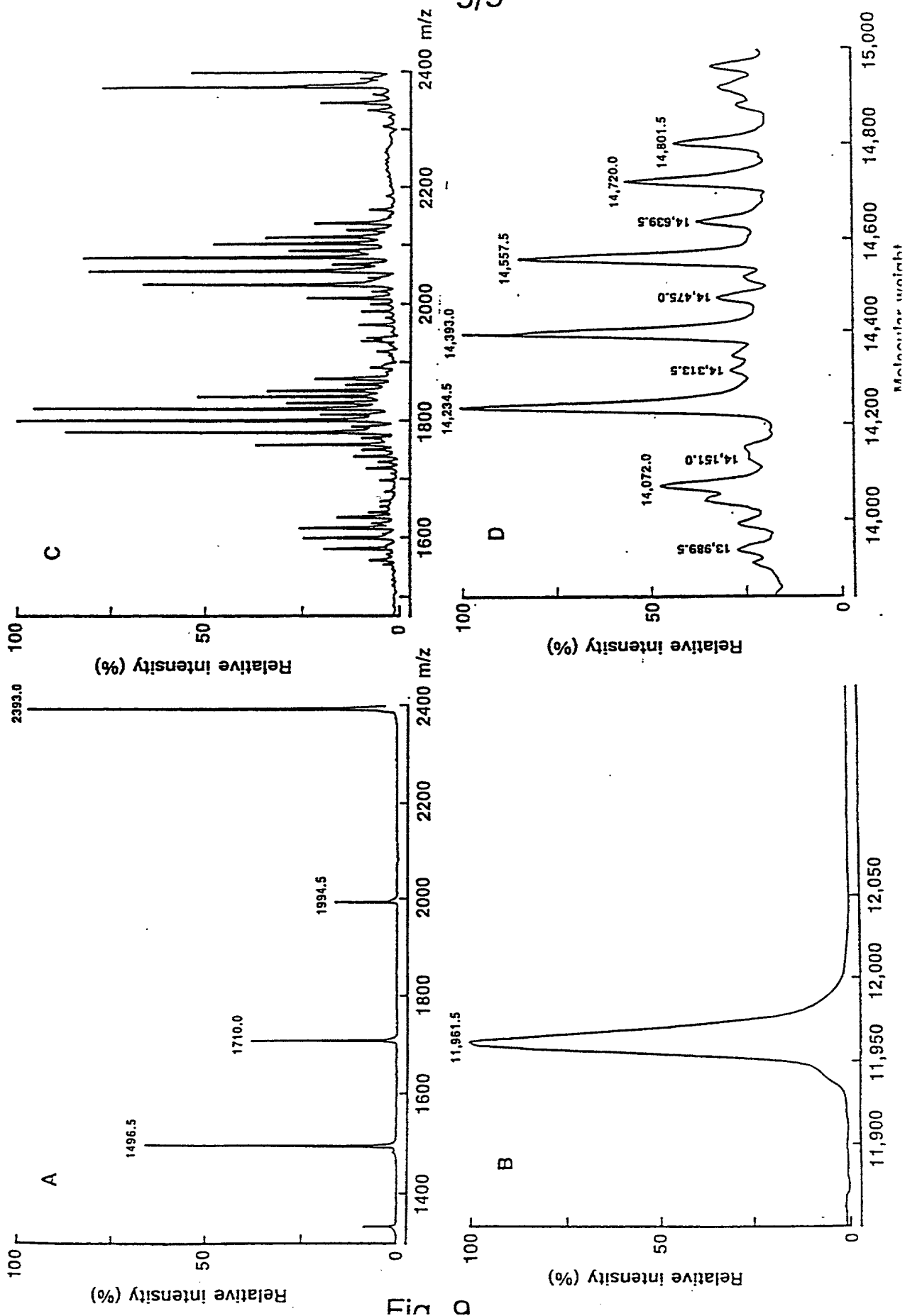


Fig. 8

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Attorney's Docket Number:

3951.204-US

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

HUMAN SPASMOLYTIC POLYPEPTIDE IN GLYCOSYLATED FORM

the specification of which (check only one item below):

[] is attached hereto

[] was filed as United States application

Serial No. _____

on _____

and was amended

on _____ (if applicable).

[x] was filed as PCT international application

Number PCT/DK94/00037

on 20 January 1994

and was amended under PCT Article 19

on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

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[illegible]

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to PCT International Applications)				Attorney's Docket Number: 3951.204-US	
I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this applications is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:					
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U.S. APPLICATIONS				STATUS (Check one)	
U.S. APPLICATION NUMBER	U.S. FILING DATE		Patented	Pending	Abandoned
PCT APPLICATIONS DESIGNATING THE U.S.					
APPLICATION NO.	FILING DATE	US SERIAL NUMBERS ASSIGNED (if any)			
PCT/DK94/00037	20 January 1994			X	
POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.					
<div style="display: flex; justify-content: space-between;"> <div>Steve T. Zelson, Esq. Reg. No. 30,335</div> <div>Elias J. Lambiris, Esq. Reg. No. 33,728</div> <div>Cheryl H. Agris Reg. No. 34,086</div> <div>Karen A. Lowney Reg. No. 31,274</div> <div>James J. Harrington Reg. No. 38,711</div> </div>					
Send Correspondence to: Steve T. Zelson, Esq. Novo Nordisk of North America, Inc. 405 Lexington Avenue, Suite 6400 New York, New York 10017				Direct Telephone Calls To: Steve T. Zelson (212) 867-0123	
	Full Name of Inventor	Family Name Thim	First Given Name Lars	Second Given Name	
	Residence & Citizenship	City DK-2820 Gentofte	State or Foreign Country Denmark	Country of Citizenship Denmark	
	Post Office Address	Post Office Address Skiftevej 22	City DK-2820 Gentofte	State & Zip Code/Country Denmark	
2	Full Name of Inventor	Family Name Norris	First Given Name Kjeld	Second Given Name	
	Residence & Citizenship	City DK-2900 Hellerup	State or Foreign Country Denmark	Country of Citizenship Denmark	
	Post Office Address	Post Office Address Ahlmanns Allé 34	City DK-2900 Hellerup	State & Zip Code/Country Denmark	
3	Full Name of Inventor	Family Name Norris	First Given Name Fanny	Second Given Name	
	Residence & Citizenship	City DK-2900 Hellerup	State or Foreign Country Denmark	Country of Citizenship Denmark	
	Post Office Address	Post Office Address Ahlmanns Allé 34	City DK-2900 Hellerup	State & Zip Code/Country Denmark	
4	Full Name of Inventor	Family Name Bjørn	First Given Name Søren	Second Given Name Erik	
	Residence & Citizenship	City DK-2800 Lyngby	State or Foreign Country Denmark	Country of Citizenship Denmark	
	Post Office Address	Post Office Address Marie Grubbes Allé 47	City DK-2800 Lyngby	State & Zip Code/Country Denmark	

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to PCT International Applications)				Attorney's Docket Number: 3951.204-US	
5	Full Name of Inventor	Family Name Christensen	First Given Name Mogens	Second Given Name	
	Residence & Citizenship	City DK-2820 Gentofte	State or Foreign Country Denmark	Country of Citizenship Denmark	
	Post Office Address	Post Office Address H.A. Clausensvej 15	City DK-2820 Gentofte	State & Zip Code/Country Denmark	
6	Full Name of Inventor	Family Name Nielsen	First Given Name Per	Second Given Name Franklin	
	Residence & Citizenship	City DK-3500 Værløse	State or Foreign Country Denmark	Country of Citizenship Denmark	
	Post Office Address	Post Office Address Jomfrubakken 3G	City DK-3500 Værløse	State & Zip Code/Country Denmark	
7	Full Name of Inventor	Family Name	First Given Name	Second Given Name	
	Residence & Citizenship	City	State or Foreign Country	Country of Citizenship	
	Post Office Address	Post Office Address	City	State & Zip Code/Country	
8	Full Name of Inventor	Family Name	First Given Name	Second Given Name	
	Residence & Citizenship	City	State or Foreign Country	Country of Citizenship	
	Post Office Address	Post Office Address	City	State & Zip Code/Country	
9	Full Name of Inventor	Family Name	First Given Name	Second Given Name	
	Residence & Citizenship	City	State or Foreign Country	Country of Citizenship	
	Post Office Address	Post Office Address	City	State & Zip Code/Country	
<p>I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.</p>					
Signature of Inventor 1 <i>Lars Thim</i>		Signature of Inventor 2 <i>Lars Nielsen</i>		Signature of Inventor 3 <i>P. Nielsen</i>	
Date 1995.07.19		Date 1995.07.21		Date 1995.07.21	
Signature of Inventor 4 <i>Ebbe Børn</i>		Signature of Inventor 5 <i>Mogens Christensen</i>		Signature of Inventor 6 <i>P. F. Nielsen</i>	
Date 1995.07.19		Date 1995.07.19		Date 1995.07.19	
Signature of Inventor 7		Signature of Inventor 8		Signature of Inventor 9	
Date		Date		Date	